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(54) Title: METHOD OF CLONAL GROWTH OF <i>STREPTOCOCCUS PNEUMONIAE</i> (57) Abstract A culture medium containing no complex animal components is useful for production of bacteria and inoculum development from which pharmaceutically important products are to be isolated.		

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TITLE OF THE INVENTION

METHOD OF CLONAL GROWTH OF STREPTOCOCCUS PNEUMONIAE

BACKGROUND OF THE INVENTION

5 Production of compounds of pharmaceutical significance by culturing bacteria and isolation of products produced in culture is an expanding field of science. An impediment to obtaining regulatory approval for products produced in this manner is encountered whenever the bacterial organism being cultured requires complex animal
10 components for its growth. For example, culture of Streptococcus pneumoniae (pneumococci), to produce pneumococcal polysaccharides for vaccine production, particularly at the inoculum development stage, is typically performed in media containing complex bovine or ovine
15 derived products such as peptones, gelatins, caseins, or blood derived products. S. pneumoniae are known to be fastidious, having nutritional requirements for choline [Rave et al., J. Bact., 40, 695-704 (1940)], vitamins [Adams et al., J. Bact., 49 401-409 (1945)], and biotin. [Bohonos and Sabborow, Arch. Biochem. 3, 257-259 (1943)]. Even
20 where subsequent culture of bacteria in media free of complex bovine or ovine derived products has been achieved, initial development of the culture inoculum of S. pneumoniae has been accomplished in blood-containing or bovine/ovine derived product media. This invention demonstrates the feasibility of inoculum development in a medium
25 devoid of these complex and potentially troublesome components.

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One commercially available medium useful for bacterial culture is known as PYG. PYG contains:

	peptone	5 g
	trypticase	5 G
5	yeast extract	10 g
	resazurin solution	4 ml
	salts solution	40 ml
	distilled water	1000 ml
	hemin solution	10 ml
10	vitamin K	0.2 ml
	cysteine HCl•H ₂ O	0.5 g
	glucose	10 g

15 (see Anaerobe Laboratory Manual, 4th Edition, 1977, Holdeman et al. eds; Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, p13). S. pneumoniae can be cultured in this medium, but since the medium contains beef peptone, it is not desirable for production of vaccine components.

20 One significant reason for using a culture medium free of animal or blood products is to reduce or avoid the possibility of contamination by agents which cause spongiform encephalopathies, such as Prion Disease [see, for example, The Lancet, vol. 336, p21-22, July 7, 1990, and references cited therein]. The etiologic agent of such
25 diseases as scrapie in animals, Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, and kuru in man, is thought to be transmissible by animal blood products, particularly bovine or ovine derived products.

We have discovered a culture medium completely devoid of complex animal derivatives which nonetheless supports the growth of the pneumococci, such as Streptococcus pneumoniae strains (Danish
30 nomenclature based on serotype) 1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. The medium does not contain bovine or ovine-derived products, nor does it contain blood. Furthermore, the inocula derived in this new medium can be used to initiate production of the pneumococcal capsular

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polysaccharide which can be harvested and used to prepare vaccines, as described for example in U.S. Patent 4,695,624.

5 The advantage of this new medium is the elimination of animal components (especially bovine or ovine derived products) from the product production process. By eliminating animal components, the dangers of contamination with reactive blood group substances or adventitious agents are reduced, and the need to remove these contaminants during purification is eliminated. It also becomes less necessary to demonstrate the absence of such contaminants in the final
10 vaccine product.

Thus, according to this invention, a new medium composition for culturing bacteria is provided. A method for using said composition, particularly for inoculum development, is also provided. The invention will allow production of fastidious bacteria such as S.
15 pneumoniae, and frozen seed stocks thereof which have not had contact with bovine or ovine derived materials. This will facilitate the regulatory review process for the pharmaceutical products derived from culture of such organisms. Thus, according to this invention, culture growth and inoculum development can be obtained in a medium
20 free of bovine or ovine derived products. Furthermore, the invention provides an alternate and preferred culture medium and method for production of inocula for pneumococcal capsular polysaccharide production.

25 SUMMARY OF THE INVENTION

A culture medium is provided which contains no bovine, ovine or crude blood derived products, but which is effective to support inoculum development for growth of Streptococcus pneumoniae and production of pneumococcal capsular polysaccharide for vaccine
30 production. In a preferred formulation of the medium, the medium comprises:
modified peptone yeast base, salt solution, resazurin, vitamin K, hemin, sodium bicarbonate, L-cysteine HCl, glucose, and HEPES buffer.

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DETAILED DESCRIPTION OF THE INVENTION

One preferred formulation of the medium of this invention contains the following ingredients per liter:

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Yeast Extract	10 g
Soy Peptone	10 g
Salt Solution	20 ml
Resazurin	1 mg
Vitamin K	0.5 mg
Hemin	5 mg
Sodium Bicarbonate	0.4 g
L-cysteine HCl	0.85 g
Glucose	10 g
HEPES buffer (pH 7.3 - 7.8)	47.66 g

This specific formulation has been named SYG medium. However, it should be understood that the very precise amounts of ingredients provided above may be optimized, or modified so long as no animal products are introduced. The key aspect of the medium is the absence of blood, bovine or ovine derived products and the ability of the medium to support growth of Streptococcus pneumoniae and thereby the production of pneumococcal polysaccharides useful for vaccine production.

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The modified Soy peptone and yeast base are hydrolysates of yeast and soy proteins. Several commercial sources for these components are available. We have found that Difco yeast extract and Sheffield HY-SOY PEPTONE are quite acceptable for this purpose.

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The salt solution may contain:

CaCl ₂ (anhydrous)	0.2 g
MgSO ₄ (anhydrous)*	0.2 g
K ₂ HPO ₄	1.0 g
KH ₂ PO ₄	1.0 g
NaHCO ₃	10.0 g
NaCl	2.0 g

(Mix CaCl₂ and MgSO₄ in 300 ml distilled water until dissolved. Add 500 ml water and, while swirling, slowly add remaining salts. Continue swirling until all these salts are dissolved. Add 200 ml distilled water, mix and store at 4°C. * Alternatively, use 0.48 g MgSO₄·7H₂O).

Other compositions for the salt solution may also be acceptable, so long as sufficient buffering and osmolarity are maintained.

The resazurin is completely optional and it may be eliminated or replaced with another indicator as desired. Its sole purpose is to indicate the redox potential of the medium. If included, it may be prepared as follows: Dissolve 25 mg resazurin in 100 ml distilled water. Resazurin is available in powder form from Fisher, Difco and Baker.

Vitamin K may be required, depending on the organism being cultured. If provided, it can be prepared as follows: Vitamin K₁ Stock Solution (to be added to media): Dissolve 0.15 ml of Vitamin K₁ in 30 ml of 95% ethanol. Do not sterilize since it is added to media before autoclaving. Add 0.02 ml of stock solution/100 ml of medium after the medium is boiled but before it is dispensed and autoclaved. Final concentration in medium = 1 nl/ml (final concentration is about 1.0 µg/ml). Keep the Vitamin K₁ stock solution under refrigeration and in the dark. Discard the stock solution after about one month.

Hemin is a necessary component for the cultivation of Streptococcus pneumoniae and may be required by other organisms, especially in a medium containing no blood products. Since hemin is a purified, low molecular weight product, absence of contaminating

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agents can be assured. It may be prepared as follows: Dissolve 50 mg hemin in 1 ml 1 N NaOH; bring to 100 ml with distilled water. Autoclave at 121°C for 15 min. Add 1 ml of this solution to 100 ml of medium.

5 Sodium bicarbonate is provided as a stimulating agent for growth of fastidious organisms.

L-Cysteine may or may not be required, depending on the organism cultured. If a well reduced medium for anaerobic culture is desired, addition of L-Cysteine will assist in maintainance of the
10 reduced environment.

Glucose is provided as a carbon source, and may be replenished upon depletion. Alternate carbon sources, such as glycerol, may also be used to advantage.

15 HEPES buffer is added to maintain pH control. Other buffers, such as Tris, may also be used, depending on the preferred pH of culture. 0.2M HEPES at a pH of about 7.7 has been found acceptable for culture of Streptococcus pneumoniae.

The foregoing description provides a basis for modifying the specific medium formulation of this invention, while maintaining the
20 key feature of being free of blood or bovine/ovine derived products. The method of using the medium of this invention comprises the steps of

- (a) preparing a stock culture on a SYG agar plate;
- (b) selecting a single colony for expansion in a culture of SYG
25 medium, and optionally preparing a frozen stock of the culture;
- (c) growth of the culture in SYG, at 37°C, for about 5-24 hours, with the length of cultivation varying according to the cultured species.

30 SYG agar is SYG medium with 20 g/L agar. Glycerol or other cryopreservatives known in the art can be added to cultures to maintain viability of frozen stocks. Preferably, glycerol is added to a final concentration between about 15 and 25%, and preferably 17%.

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The following Examples, demonstrate production and use of the medium of this invention to support the inoculum development and growth of Streptococcus pneumoniae of various serotypes, and the production by those cultures of polysaccharide. The polysaccharide thus produced is useful for the preparation of conjugate vaccines, such as those described in U.S. Patent 4,695,624, herein incorporated by reference. Methods for quantitating pneumococcal polysaccharide, including immunodiffusion and radioimmunoassay are well known in the art (see for example Heidelberger, J. Exp. Med., 55, p555 (1932); Ouchterlony, Handbook of Immunodiffusion and Immunoelectrophoresis, Ann Arbor Publishers (1968); EP 0 497 525 A2).

EXAMPLE 1

Growth Of S. Pneumoniae 9V

This example demonstrates development of a medium devoid of bovine or ovine derived products which supports good growth of Streptococcus pneumoniae strain 9V. Previous work resulted in growth of S. pneumoniae 9V in the commercially available PYG medium. For this example, soy peptone was used in place of animal peptone and trypticase to make modified PYG medium, which was otherwise identical to PYG medium. One lot of this medium, referred to as W001, was prepared using Sheffield HySoy peptone. A second lot, referred to as W002, was prepared using Deltown SE50M soy peptone. S. pneumoniae 9V from identical frozen stocks was inoculated into both formulations. Culture tubes were incubated at 37°C for 14 hours. Both formulations supported growth of S. pneumoniae 9V. However, the Sheffield HySoy formulation supported much heavier growth than did the Deltown SE50M formulation. Further details of this work are presented below:

Modified PYG (W001) - HySoy-100 µL inoculum from a frozen vial.

Modified PYG (W002) - Deltown-100 µL inoculum from a frozen vial.

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Liquid Mod. PYG-HySoy 0 - No growth
100 μ l - Heavy Growth, Turbid, Uniform,
no sediment

5 Liquid Mod. PYG-Deltown 0 - No growth
100 μ l - very light growth 1/10 of Hy-Soy

To expand upon these observations the cultures grown in
liquid broth were inoculated into #W001 and #W002 for sub-culture
and determination of the best liquid formulation:

10

#W001 (HySoy)#W002 (Deltown)InoculatedInoculated

10 μ l 100 μ l 10 μ l 100 μ l
into

10 μ l 100 μ l 10 μ l 100 μ l
into

15 #W001 #W001 #W002 #W002 #W001 #W001 #W002 #W002

MEDIUM

	Inoculum Source	#W001	#W002	Result
20	HySoy liquid 10 μ l	+		good growth
	HySoy liquid 100 μ l	+		good growth
	HySoy liquid 10 μ l		+	very faint turbidity
	HySoy liquid 100 μ l		+	very faint turbidity
25	Deltown liquid 10 μ l	+		good growth
	Deltown liquid 100 μ l	+		good growth
	Deltown liquid 10 μ l		+	faint growth
	Deltown liquid 100 μ l		+	light growth

30

Conclusions/Results

1. Liquid to liquid transfer was successful for 9V growth.
2. In all cases, media prepared with Hy-Soy provides more luxuriant, more turbid growth than the media with the Deltown SE50m soy peptone.

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The pH of the #W001 media is about 6.7 and #W002 is about 6.65. It is likely that a higher pH (about 7.5) with buffering, eg. HEPES at 0.1 M or 0.2 M would support even better growth than the media without buffering.

5

EXAMPLE 2

Production Of S. Pneumoniae 9V Extracellular Polysaccharide

The medium "SYG" supports excellent growth of Streptococcus pneumoniae 9V. The "SYG" uses Sheffield Hy-Soy peptone in place of peptone and trypticase, and contains 0.2 M HEPES.

In this example, 9V was cultured in PYG as a positive control, with and without HEPES, pH about 7.5 and SYG with and without HEPES pH about 7.5 and the culture broth was assayed for production of S. pneumoniae 9V polysaccharide (Pn9VPs) using immunodiffusion and rate nephelometry.

Materials: S. pneumoniae 9V glycerol seed stock; 1M HEPES buffer pH 7.70 sterile filtered; PYG, Scott 3100-4807, #R7320, (about 4.75 ml/tube); SYG (mod. PYG); #W001 (~4.6 ml/tube); Phenol; Columbia Blood Agar Plates (CBA).

Method: 10 µl of S. pneumoniae 9V inoculum was added to tubes 5, 10, 11 and 12, mixed well and incubated in a GasPak jar with CO₂, at 37° for 10 hrs.

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Tube #	Media	9V inco. 10μL	0.2M HEPES	vol IM HEPES	phenol	phenol μL tube	pH pre inoc.	pH final	A600 pre inoc.	A600 final	visual observation
1	PYG	-	-	-	-	-	6.7		.059		
2	PYG	-	-	-	+	56	6.7		.059		
3	PYG	-	+	.95 ml	-	-	7.52		.068		
4	PYG	-	+	.95 ml	+	66	7.52		.068		
11	PYG	+	-	-	+	56	6.7	6.7	.060	.060	no visible growth
5	PYG	+	+	.95 ml	+	66	7.52	6.63	.068	4.31	heavy growth, not as dense as #10
6	SYG	-	-	-	-	-	6.6		.128		
7	SYG	-	-	-	+	56	6.6		.128		
8	SYG	-	+	.92 ml	-	-	7.46		.120		
9	SYG	-	+	.92 ml	+	66	7.46		.120		
12	SYG	+	-	-	+	56	6.7	5.05	.128	0.95	light-fair growth
10	SYG	+	+	.92 ml	+	66	7.46	5.38	120	6.03	very dense growth some sediment

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The tubes were examined for growth, and the pH, A600 were measured. Phenol was added to each tube, mixed well and transferred to clean 50 ml sterile Oak Ridge tubes. The tubes were set to shake at 200 rpm, at 37°C for 2 hrs.

5 After 2 hrs kill with phenol, (Note: post phenol kill- 200 ml from 1:100 phenol killed broth was pipetted onto the surface of 2 CBA plates per sample. The plates were incubated at 37°C under CO₂ overnight. No growth on CBA plates indicated complete killing by phenol). The tubes were centrifuged in an SS-34 rotor at 10,000 rpm, 10 4°C for 20 minutes. The supernatant was removed into 4X2 ml Wheaton vials for each sample. The vials were stored at 4°C. Sample numbers 1-4 and 6-9 were mixed with the appropriate amounts of HEPES and/or phenol, mixed well and aliquotted into 4X2 ml Wheaton vials.

15 The CBA plates from phenol killed broth, samples #5, and #10 showed no growth. A 1 ml aliquot of 4°C stored supernatant from samples # 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 was assayed for Pn 9V Ps by immunodiffusion.

20 Pn 9V standard: 1.0 mg/ml and diluted to 0.5, 0.25, 0.125, 0.0625, 0.031, 0.0155 mg/ml. Intense precipitin bands were observed at each well.

Sample numbers 1, 2, 3, and 4 were media controls. No reactivity with Pn 9V antibody was seen.

25 Sample number 5 was loaded undiluted (st), and at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 dilutions. Weak bands were seen at the undilute and 1:2 positions. The concentration of sample #5 corresponds to a band intensity of ~0.05 mg/ml compared with 9V standard.

Sample numbers 6, 7, 8 and 9 were media controls. No reactivity with 9V antibody was seen.

30 Sample 10 is 9V grown in SYG plus HEPES. A strong band in samples diluted to 1:8 corresponds to a polysaccharide concentration of ~ 0.25 mg/ml compared to 9V standard.

Sample No. 12 gave an immunodiffusion response corresponding to about 0.05 mg/ml.

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All samples tested by immunodiffusion were phenol killed supernatants stored at 4°C.

The same panel of samples was also analyzed by a Pn 9V Ps rate nephelometry assay.

Conclusions:

1. All media controls were non reactive.
2. Samples 5, 10, and 12, both liquid and frozen, respond positively in the assay.
3. Samples No. 5, 10 and 12 have very different rates of response in a rate nephelometry assay. There may be several reasons for this:
 - a. The 9V standard is a purified and sized 9V Ps, while the PnPs in the broth is crude, not purified or sized.
 - b. The organisms may be making different sized Ps depending upon pH, A600, growth rate, time in stationary phase, etc. Therefore, Pn 9V Ps quantitation is not possible yet due to the above factors.

Results:

Tube No.	Immunodiffusion Results	Rate Nephelometry Results
1	No reaction with Ab	No reaction
2	No reaction with Ab	No reaction
3	No reaction with Ab	No reaction
4	No reaction with Ab	No reaction
11	Not submitted	Not submitted
5	pos. ~0.05 mg/ml	positive liquid & -70°C samples
6	No reaction with Ab	No reaction
7	No reaction with Ab	No reaction
8	No reaction with Ab	No reaction
9	No reaction with Ab	No reaction
12	pos. ~0.05 mg/ml	positive liquid & -70°C samples
10	pos. ~0.05 mg/ml	positive liquid & -70°C samples

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EXAMPLE 3

Preparation Of S. Pneumoniae Clonally Isolated Master Cultures:

5 We have prepared lab stocks in SYG of S. pneumoniae 4, 6B, 9V, 14, 18C, 19F and 23F serotypes for routine lab use. They were prepared as described below.

Preparation Of Master Lab Working Stock:

10 Lyophilized cultures of S. pneumoniae were resuspended in beef heart infusion broth, inoculated into SYG liquid, and grown 6-12 hrs. Glycerol was added, and the culture was aliquoted and frozen. We made ~5 vials of each serotype. These vials are designated to be master lab working stocks. These cultures were subsequently expanded in SYG
15 broth to make ~60 vials for each serotype.

Expansion And Preparation Of Mock Premaster Cultures:

A vial of S. pneumoniae frozen master lab working stock was thawed and inoculated into SYG broth and grown 6-12 hrs.
20 Glycerol was added and the cultures aliquoted and frozen to give 60 vials for each serotype. These cultures have served as our routine lab culture source. To prepare clonally isolated pre-master cultures, a lyophilized vial of S. pneumoniae is resuspended in SYG and streaked onto SYG agar. After incubation for 12-26 hours at 37°C under CO₂ a
25 single colony was picked and streaked onto SYG agar. Plates were incubated 12-26 hours at 37°C under CO₂ and a single colony was picked and inoculated into SYG liquid, and grown 6-12 hrs. Glycerol was added and the culture was aliquotted and frozen.

EXAMPLE 4

30 Growth Of S. Pneumoniae 9V And Polysaccharide Production In A Fermentor:

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We have used our premaster culture of Pn 9V to inoculate 350 ml of SYG liquid. This was used to inoculate 15-L of S. pneumoniae production medium. Culture growth and polysaccharide production was equivalent to that seen when S. pneumoniae 9V was derived from blood derived cultures.

EXAMPLE 5

Growth Of S. Pneumoniae Of Other Subtypes:

Culture of any of the Streptococcus pneumoniae strains (Danish nomenclature based on serotype) 1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F in SYG medium and inoculum development is conducted essentially as described above for S. pneumoniae 9V. Each of Streptococcus pneumoniae 4, 6B, 14, 18C, 19F, and 23F were grown in SYG and positive immunodiffusion and RIA data for pneumococcal polysaccharide production was obtained. Each serotype was grown in SYG at 37°C under CO₂ for 8-24 hours. Samples were taken during growth, cells were inactivated with phenol, and supernatants were assayed for extracellular polysaccharide by immunodiffusion and quantitated by RIA:

Serotype	Hours Growth	Start A600	End A600	Immuno-diff.	RIA µg/mL
4	24	0.086	1.68	+	140
6B	24	0.086	2.21	+	370
9V	24	0.086	1.76	+	121
14	24	0.086	3.69	+	68
18C	8	0.086	3.98	+	240

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Serotype	Hours Growth	Start A ₆₀₀	End A ₆₀₀	Immuno- diff.	RIA μg/mL
19F	8	0.086	3.96	+	161
23F	12	0.086	2.71	+	317

Based on these results, it is expected that all pneumococcal serotypes will grow adequately in the SYG medium or a modification thereof, according to this invention, and that inoculum development of pneumococcal serotypes in this medium is practical.

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WHAT IS CLAIMED IS:

1. A medium for inoculum development or growth of Streptococcus pneumoniae which contains no bovine, ovine or crude blood derived products.
2. The medium of Claim 1 which comprises: modified peptone yeast base, salt solution, resazurin, vitamin K, hemin, sodium bicarbonate, L-cysteine HCl, glucose, and HEPES buffer.
3. The medium of Claim 3 which comprises, per liter,:
- | | |
|---------------------------|----------|
| Yeast Extract | 10 g |
| Soy peptone | 10 g |
| Salt Solution | 20 ml |
| Resazurin | 1 mg |
| Vitamin K | 0.5 mg |
| Hemin | 5 mg |
| Sodium Bicarbonate | 0.4 g |
| L-cysteine HCl | 0.85 g |
| Glucose | 10 g |
| HEPES buffer (pH 7.3-7.8) | 47.66 g. |
4. A method of culturing Streptococcus pneumoniae in the medium of Claim 3.
5. The method of Claim 4 which comprises:
- preparing an SYG agar plate of Streptococcus pneumoniae;
 - selecting a single colony for expansion in a culture of the SYG medium, and optionally storing the culture as a frozen glycerol stock; and
 - growth of the culture in step (b), at 37°C, for about 5-24 hours.

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6. A method for preparing an isolate of Streptococcus pneumoniae from the single colony stage in a medium containing no bovine derived, ovine derived or crude blood derived products which comprises:

- 5 (a) preparing an agar plate of said medium streaked with said Streptococcus pneumoniae, wherein said agar plate comprises agar, Yeast Extract, Soy Peptone, salts to maintain buffering and osmolarity, Vitamin K if known to be required for the particular strain being isolated, Hemin, Sodium Bicarbonate, L-cysteine HCl, Glucose, a buffer to maintain the pH at between about pH 7.3-7.8, but no bovine derived, ovine derived or crude blood-derived
- 10 products; and
- (b) selecting a single colony for expansion in a culture of the SYG medium absent the agar, and optionally storing the culture as a frozen glycerol stock.

7. The method of Claim 6 wherein the medium in step
- 15 (a) is an SYG agar plate streaked with said Streptococcus pneumoniae, wherein the SYG agar plate contains, on a per liter basis, about 20 g of agar and:

	Yeast Extract	10 g
	Soy Peptone	10 g
20	Salt Solution	20 mL
	Resazurin	1 mg
	Vitamin K	0.5 mg
	Hemin	5 mg
	Sodium Bicarbonate	0.4 g
	L-cysteine HCl	0.85 g
25	Glucose	10 g
	HEPES Buffer (pH 7.3-7.8)	47.66g; and

wherein said salt solution contains CaCl₂ (0.2 g anhydrous), MgSO₄ (0.2 g anhydrous), K₂HPO₄ (1.0 g), KH₂PO₄ (1.0 g), NaHCO₃ (10 g), NaCl (2 g).

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AMENDED CLAIMS

[received by the International Bureau on 17 February 1994 (17.02.94);
original claims 1-7 cancelled;
new claims 1 and 2 added (2 pages)]

1. A method for clonal growth of Streptococcus pneumoniae from the single colony stage in a medium containing no
5 bovine derived, ovine derived or crude blood derived products which comprises:
(a) preparing an agar plate of said medium streaked with said Streptococcus pneumoniae, and growing said Streptococcus pneumoniae,
wherein said agar plate comprises agar, Yeast Extract, Soy Peptone,
10 salts to maintain buffering and osmolarity, Vitamin K if known to be required for the particular strain being isolated, Hemin, Sodium Bicarbonate, L-cysteine HCl, Glucose, a buffer to maintain the pH at between about pH 7.3-7.8, but no bovine derived, ovine derived or crude blood-derived products; and
15 (b) selecting a single colony for expansion by growing in a culture of the same medium used in step (a) absent the agar, and optionally storing the culture as a frozen glycerol stock.

2. The method of Claim 1 wherein the medium in step
20 (a) is an SYG agar plate streaked with said Streptococcus pneumoniae, wherein the SYG agar plate contains, on a per liter basis, about 20 g of agar and:

25	Yeast Extract	10 g
	Soy Peptone	10 g
	Salt Solution	20 mL
	Resazurin	1 mg
	Vitamin K	0.5 mg
	Hemin	5 mg
30	Sodium Bicarbonate	0.4 g
	L-cysteine HCl	0.85 g
	Glucose	10 g
	HEPES Buffer (pH 7.3-7.8)	47.66g; and

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wherein said salt solution contains CaCl_2 (0.2 g anhydrous), MgSO_4 (0.2 g anhydrous), K_2HPO_4 (1.0 g), KH_2PO_4 (1.0 g), NaHCO_3 (10 g), NaCl (2 g).

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STATEMENT UNDER ARTICLE 19

The newly presented claims more clearly define the invention being claimed and bring these claims into concordance with allowed claims in the United States.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/09584

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 1/20

US CL : 435/252.1, 253.1, 253.4, 253.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.1, 253.1, 253.4, 253.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,355,111 (SHIMIZU ET AL.) 19 OCTOBER 1982, SEE ENTIRE DOCUMENT.	1-7
Y	US, A, 4,686,102 (RITCHEY ET AL.) 11 AUGUST 1987, SEE ENTIRE DOCUMENT.	1-7
Y	AMERICAN TYPE CULTURE COLLECTION, CATALOGUE OF BACTERIA AND PHAGES, 17TH ED., ISSUED 1989, PAGES 334 AND 339, SEE ENTIRE DOCUMENT.	1-7
Y	CANO ET AL., "ESSENTIALS OF MICROBIOLOGY", PUBLISHED 1988 BY WEST PUBLISHING COMPANY (ST. PAUL), PAGES 88-89, SEE ENTIRE DOCUMENT.	1-7

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 December 1993

Date of mailing of the international search report

11 JAN 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

DEBORAH K. WARE

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09584

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BACTERIOLOGY, VOLUME 40, ISSUED 23 MAY 1940, RANE ET AL., "NUTRITIONAL REQUIREMENTS OF THE PNEUMOCOCCUS 1. GROWTH FACTORS FOR TYPES I, II, V, VII, VIII", PAGES 695-704, SEE ENTIRE DOCUMENT.	1-7
Y	JOURNAL OF BACTERIOLOGY, VOLUME 49, ISSUED 04 DECEMBER 1944, ADAMS ET AL., "A PARTIALLY DEFINED MEDIUM FOR CULTIVATION OF PNEUMOCOCCUS", PAGES 401-409, SEE ENTIRE DOCUMENT.	1-7
Y	ARCH. BIOCHEM., VOLUME 3, ISSUED 09 SEPTEMBER 1943, BOHONOS ET AL., "THE REQUIREMENT OF BIOTIN FOR THE GROWTH OF PNEUMOCOCCI", PAGES 257-259, SEE ENTIRE DOCUMENT.	1-7
Y	JOURNAL OF CLINICAL MICROBIOLOGY, VOLUME 11, NUMBER 1, ISSUED JANUARY 1980, BROOKS ET AL., "RAPID DIFFERENTIATION OF MAJOR CAUSATIVE AGENTS OF BACTERIAL MENINGITIS BY USE OF FREQUENCY-PULSED ELECTRON CAPTURE GAS-LIQUID CHROMATOGRAPHY: ANALYSIS OF ACIDS", PAGES 45-51, SEE ENTIRE DOCUMENT.	1-7
Y	JOURNAL OF BACTERIOLOGY, VOLUME 43, ISSUED 07 JULY 1941, BERNHEIMER ET AL., "AN IMPROVED MEDIUM FOR THE CULTIVATION OF HEMOLYTIC STREPTOCOCCUS", PAGES 495-498, SEE ENTIRE DOCUMENT.	1-7
Y	JOURNAL OF GENERAL MICROBIOLOGY, VOLUME 27, ISSUED 1962, HOLT, "THE CULTURE OF STREPTOCOCCUS PNEUMONIAE", PAGES 327-330, SEE ENTIRE DOCUMENT.	1-7
Y	JOURNAL BACTERIOLOGY, VOLUME 43, ISSUED 1942, BERNHEIMER ET AL., "FACTORS NECESSARY FOR MASSIVE GROWTH OF GROUP A HEMOLYTIC STREPTOCOCCUS", PAGES 481-494, SEE ENTIRE DOCUMENT.	1-7
Y	JOURNAL OF BACTERIOLOGY, VOLUME 74, ISSUED 1957, HOEPRICH, "EVALUATION OF AN IMPROVED CHEMICALLY DEFINED MEDIUM FOR THE CULTURE OF DIPLOCOCCUS PNEUMONIAE", PAGES 587-590, SEE ENTIRE DOCUMENT.	1-7

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US93/09584

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF PATHOLOGY AND BACTERIOLOGY, VOLUME 37, ISSUED 1933, WRIGHT, "THE IMPORTANCE OF ADEQUATE REDUCTION OF PEPTONE IN THE PREPARATION OF MEDIA FOR THE PNEUMOCOCCUS AND OTHER ORGANISMS", PAGES 257-282, SEE ENTIRE DOCUMENT.	1-7

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